Platelet Function Determined by Flow Cytometry: New Perspectives?

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Abstract

Flow cytometry enables studies of several different aspects of platelet function in response to a variety of platelet agonists. This can be done using only a small volume of whole blood, and also in blood with low platelet counts. These properties, together with the increasing number of flow cytometers available in hospitals worldwide, make flow cytometry an interesting option for laboratories interested in studies of platelet function in different clinical settings. This review focuses on practical issues regarding the use of flow cytometry for platelet function testing. It provides an overview of available activation markers, platelet agonists, and experimental setup issues. The review summarizes previous experience and factors important to consider to perform high-quality platelet function testing by flow cytometry. It also discusses its current use and possibilities and challenges for future use of flow cytometry in clinical settings.

Keywords
- blood platelets
- flow cytometry
- platelet activation
- platelet function testing

Mild bleeding disorders caused by inherited platelet dysfunction are a diagnostic challenge.[1] At the same time, inhibition of platelet activation is of paramount importance in cardiovascular diseases. In light of this, there are multiple clinical reasons to measure platelet function. The "gold standard" since the 1960s is light transmission aggregometry on citrated platelet-rich plasma (PRP), but this method is cumbersome and results vary with the platelet count.[2] In addition, it requires a relatively large volume of blood and only investigates one aspect of platelet function, the aggregation potential. Improved methods for investigation of different aspects of platelet function are therefore in demand.

In flow cytometry, single cells are passed through one or more laser beams, where the light scatter (indicating cell size and granularity) and signal intensity from different fluorescently labeled markers bound to the cell can be measured. This enables studies of several different aspects of platelet function in response to a variety of platelet agonists. The possibility to obtain results from thousands of individual platelets in just a few seconds and from just a few microliters of blood is also attractive, especially for pediatric patients. Another major advantage is that platelet function can be investigated in blood from patients with thrombocytopenia, as the technique does not require a certain cell count to give reliable results. Due to the versatility of flow cytometers, these instruments are now available in many hospitals for other applications. Thus, flow cytometry may be an attractive option for many laboratories wanting to perform platelet function testing.

This review focuses on practical issues regarding the use of flow cytometry for platelet function testing. It discusses its possibilities and limitations and reflects on its current and potential future use in clinical practice.

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Methodological Issues

Preparation of Platelets

Due to the size difference as compared with other blood cells, platelets are easily distinguishable in whole blood samples. This allows platelet analysis by flow cytometry in diluted whole blood. Although it is possible to use PRP or platelet suspensions as well, the diluted whole blood method first described by Shattil and colleagues in 1987[3] offers several advantages, as it reduces the risk of preactivation and loss of larger/denser platelet populations (during, e.g., centrifugation to produce PRP).[4,5] For some assays, the presence of other blood cells is also needed, such as when platelet–leukocyte aggregates are analyzed.

The most common choice for blood anticoagulation is still sodium citrate (3.2 or 3.8%), but the use of other anticoagulants are increasing, such as hirudin, D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK),[6] corn trypsin inhibitor, and other options, that do not interfere with the calcium levels of the blood. Strong calcium chelators such as ethylenediaminetetraacetic acid (EDTA) should be avoided as they dissociate the fibrinogen receptor glycoprotein (GP) IIb/IIIa irreversibly and thereby prevent fibrinogen binding[7] (although this can also be used to create a negative control for this analysis[8]). Something worth to keep in mind when comparing results from platelets in different anticoagulants is that platelet responses seem to be higher in platelets exposed to citrate anticoagulation, despite a large dilution of the anticoagulant in the reaction mixture where platelets are activated (see ►**Fig. 1A–C**). This is not unique for flow cytometry, and it has previously been reported that the potency of different GPIIb/IIIa antagonists differed depending on the anticoagulant used in the assay.[9,10] In addition, an enhanced thromboxane production in conditions with low extracellular calcium levels has been suggested to cause a second wave of aggregation in response to adenosine diphosphate (ADP) in citrated plasma, something that was not observed at physiological levels of calcium.[11–13] The problem with thrombin inhibitors such as hirudin and PPACK is that these make it impossible to use thrombin for platelet activation. If thrombin is used, citrate anticoagulation is the common choice. Addition of the fibrin polymerization inhibitor peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP)[14–17] to the reaction mixture is necessary if whole blood or PRP is to be used together with thrombin. With GPRP it is possible to avoid fibrin network formation, which will otherwise trap the platelets and potentially clog the flow cytometer.

For heparin as anticoagulant, there are reports stating that it either potentiates[18] or inhibits[19] platelet aggregation responses, and one flow cytometry study reporting a higher platelet response when heparin and citrate were present, as compared with citrate alone.[20] Thus, the evidence is inconclusive to the potential effects of heparin as anticoagulant. In ►**Fig. 1A–C**, platelet responses in heparin were mostly intermediate to those in citrate and hirudin. We thus suggest that if heparinized blood is the only available option, it can be used if proper controls showing no signs of preactivation are included and these are presented together with the results. It should also be kept in mind that the potential heparin-induced effects discussed here are completely unrelated to the dramatic immunological reaction leading to platelet binding and clearance in patients with heparin-induced thrombocytopenia.

For blood collection, the general recommendations are to use minimal stasis and a large needle (19–21 gauge) to ensure a smooth blood flow and to discard the first portion of blood (at least 2 mL) to avoid preactivation of platelets and inclusion of vessel wall material.[21,22] Systematic mixing (by 6–10 times full inversion) of the tube after the blood draw is important to allow rapid exposure to the anticoagulant. After that, vigorous shaking (e.g., pneumatic tube transport) or temperature shifts during transportation to the laboratory have to be avoided, as these procedures have been shown to cause platelet preactivation.[23,24] By including a sample checking for spontaneous platelet preactivation in the flow cytometry panel, it will be possible to find and potentially discard data that are unreliable due to preanalytical activation of the collected platelets (discussed further below). Blood samples should be kept at room temperature prior to analysis,[22] and actions taken to prevent temperature fluctuations. Both refrigeration and storage at 37°C affect platelet function[25] and should therefore be avoided.

With regard to the time from blood collection until the start of platelet function testing, not much data are available. Most published flow cytometry protocols for research state that preparation for testing was started "immediately" after blood collection. However, this is naturally difficult to achieve in clinical settings, and in worst case, differences in time between sampling and preparation in the control group and the patient material could be a possible cause of observed differences. Therefore, it is very important to standardize both blood sampling conditions and time to function testing, and to choose a time interval achievable for all samples. There are reports stating that the platelet responses during the first hour after blood collection in citrate tubes are actually lower, but to varying degrees, and that a higher and more stable response is achieved in samples 1 to 2 hours after blood collection.[26] For a clinical study, this time interval may be a more reasonable and achievable target window, but it should be validated for the specific protocol used in the study.

Sample Preparation

To avoid platelet aggregation, it is necessary to dilute the blood prior to platelet activation; a dilution of 1:12 to 1:13 was used in the original whole blood protocol.[3] It is also important to avoid vigorous mixing (use only brief low-speed vortex or finger tapping, with no mixing during activation). To check for potential platelet aggregation during the activation procedure, a simple way to get a crude estimate is to count the time it takes to achieve the selected number of platelets during acquisition in activated versus buffer-treated samples.[27] Although most flow cytometer brands do not have exact volumetric dispensing, this approach will detect if a significant number of platelets are lost from the single platelet gate upon activation. If this is the case, the dilution, mixing, or activation part of the protocol might need to be adjusted.

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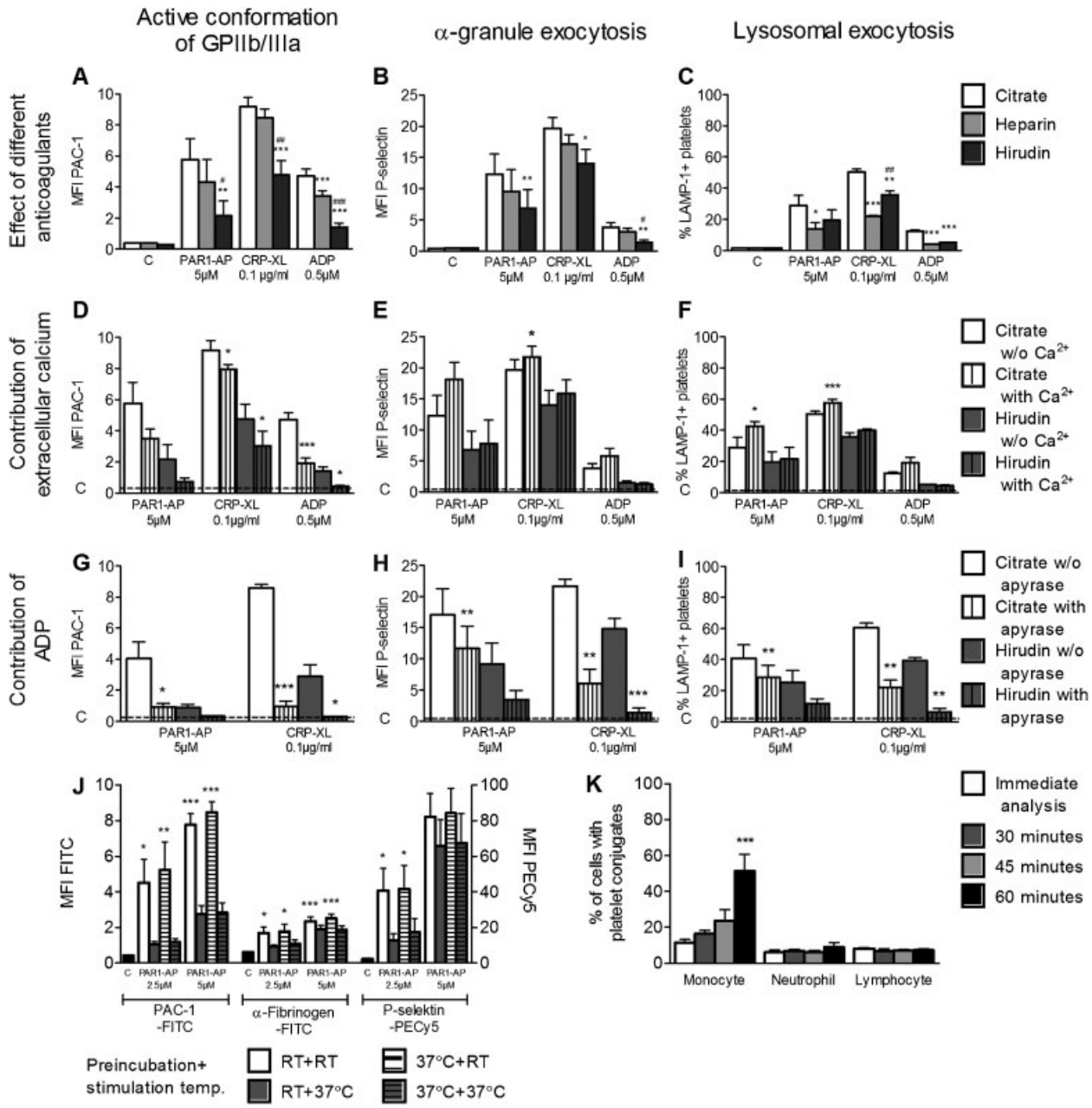

Fig. 1 Experimental conditions affecting results of platelet function testing by flow cytometry. Platelets from whole blood (1:12 dilution) were activated with Protease-activated-receptor 1-activating peptide (PAR1-AP), cross-linked collagen-related peptide (CRP-XL), adenosine diphosphate (ADP), or vehicle control (C) for 10 minutes at room temperature (RT) unless otherwise indicated. Data are presented as mean ± standard error of the mean, $n = 4$ to 5. For statistical comparisons, repeated measures ANOVA followed by Bonferroni multiple comparison test was used. Statistically significant differences are indicated by * or # if $p < 0.05$, ** or ## if $p < 0.01$, and *** or ### if $p < 0.001$. (**A–C**) Effect of different anticoagulants (citrate, heparin, and hirudin) on platelet activation responses. Median fluorescence intensity (MFI) of PAC-1 (A), P-selectin (B), and % of platelets exposing lysosomal-associated membrane protein-1 (LAMP-1) (C). */# compared with the corresponding citrate/heparin samples. (**D–F**) Effect of the absence/presence of 1.5 mM calcium (Ca^{2+}) in the buffer on platelet activation. MFI of PAC-1 (D), P-selectin (E), and % of platelets exposing LAMP-1 (F). * compared with the corresponding sample without Ca^{2+}. (**G–I**) Effect of autocrine ADP on platelet activation. ADP was degraded by addition of apyrase (grade VII, 0.25U/ml). MFI of PAC-1 (G), P-selectin (H), and % of platelets exposing LAMP-1 (I). * compared with the corresponding sample without apyrase. (**J**) Effect of temperature on the MFI of PAC-1, an anti-fibrinogen antibody and P-selectin. The antibody mixture was preincubated at RT or 37°C (10 minutes). Platelet activation with PAR1-AP was then performed at RT or 37°C. * compared with the corresponding sample activated at 37°C. (**K**) Percentage of leukocyte–platelet aggregates (detected as % of leukocytes positive for a GPIIb-antibody) at different time points after blood collection. * compared with the corresponding sample analyzed immediately.

The "Consensus protocol for flow cytometric characterization of platelet function," published by the European Working Group on Clinical Cell Analysis,[28] recommends the use of a phosphate-buffered saline buffer. Addition of bovine serum albumin is suggested to prevent nonspecific antibody binding. A modified Tyrode buffer is suggested for studies of platelet activation, as it also contains glucose and magnesium. We, and others, have good experience with the HEPES buffer described by Shattil et al,[29] which fulfill all these criteria.

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Except for cases where the intention is to study the loss of surface receptors due to internalization,[27,30] it is common and convenient to add the antibodies to the initial sample dilution mixture prior to activation. To minimize issues with unspecific binding, which might reduce the dynamic range of the assay, all antibodies should be titrated to find the lowest concentration needed to fully saturate a highly activated platelet sample.[31] This concentration is often lower than the one suggested by the manufacturer, as platelets are rarely the primary target for commercial antibodies. For studies of platelet activation, it is also absolutely essential to be able to tell where the gate dividing positive and negative platelets should be placed. Although the use of isotype controls is much discussed,[31,32] our experience is that an isotype control where the dilution has been based both on antibody concentration and on knowledge regarding the number of fluorophores per protein (F/P ratio) usually nicely mirrors the peak in the resting sample with only buffer added, except for the right-hand shift caused by preactivated platelets. It needs to be confirmed that this is the case; otherwise, the isotype control is useless for gating. It should be noted that both the concentration and F/P ratio commonly vary between different batches of antibodies, especially for small fluorophores such as fluorescein isothiocyanate. Thus, if not stated on the inserts, this information needs to be requested from the manufacturers to make a good control for gating.

The plasma concentration of biologically available calcium (free ionized calcium) is ~1.2 mM[33] and constitutes the physiological concentration of extracellular calcium for platelets. Despite this, many in vitro platelet experiments are performed in buffers without added calcium. Addition of calcium to the buffer may affect the way the platelets respond to agonists, as influx of extracellular calcium is part of the physiological response to activation,[34] for example, important for generation of procoagulant platelets.[35,36] ►**Fig. 1D–F** shows that transformation of the fibrinogen receptor GPIIb/IIIa into its high-affinity state was somewhat inhibited in the presence of extracellular calcium, perhaps related to the downregulation of GPIIb/IIIa in highly activated platelets.[37] On the other hand, both P-selectin and lysosomal-associated membrane protein-1 (LAMP-1) exposure were increased in the presence of calcium, showing that even though the intracellular calcium stores are sufficient to enable granule exocytosis,[38] this process is enhanced in the presence of extracellular calcium. In some cases, the addition of calcium is absolutely necessary, such as when using annexin V to detect the exposure of phosphatidylserine (PS)[39–41] or when studying the assembly of coagulation factor complexes on the platelet surface.[42]

Identification of Platelets

As compared with standard flow cytometry settings, such as those for leukocyte analysis, platelet protocols use logarithmic scaling for forward and side scatter detectors. In addition, the standard threshold in forward scatter applied for leukocytes needs to be significantly decreased, as platelet-sized particles are normally excluded as debris in standard flow cytometry protocols. The small size of platelets renders them detectable by their forward/side scatter characteristics alone, but in the clinical setting, where disturbed cells of other origins might occur, inclusion of a platelet identification marker is clearly warranted. Common antibodies used to identify platelets target GPIb, GPIX, or the GPIIb/IIIa complex.[43] However, in the latter case, the antibody used must be able to bind both the active and resting form of the receptor and not interfere with binding of fibrinogen/conformation-sensitive antibodies, if such markers are also included in the protocol. Platelets can then be identified by fluorescence from the identification marker together with one of the scatter parameters, collecting data from at least 5,000 to 10,000 platelets. Subsequent gating to exclude nonsingle events is recommended as a next step. If these events are frequent and caused by coincidence,[44] where multiple particles pass the laser together, further dilution may help.

One potentially interesting feature in the platelet activation response is the release of platelet microparticles upon activation. Many reports have been published where microparticles formed in vivo are detected, and recent reviews report findings in numerous pathological conditions.[45,46] As this review focuses on platelet function testing, studies of microparticles alone will not be discussed. However, for all types of studies on microparticles, it is important to note that the use of beads for size determination is not accurate, as the refractive indexes of polystyrene beads and cell fragments differ.[47] Due to antigen depletion on smaller sized particles, markers with low surface density may not be detectable on microparticles by flow cytometry.[48] There is also a lower limit for the size of particles possible to detect by flow cytometry, and many particles fall below this limit (the "iceberg theory") or will be counted together as one larger particle ("swarm detection").[49–51] Thus, the majority of detected particles with standard protocols have been suggested to be "smaller platelets" rather than microparticles.[52] However, the use of a fluorescence threshold on a highly expressed marker (e.g., GPIIb/IIIa) instead of a scatter threshold enables detection of smaller platelet-derived particles.[53,54] It is also to be noted that separation of particles based on their size is better accomplished using forward scatter on some brands of flow cytometers, while others brands give better size separation using the side scatter parameter.[55]

Platelet Activation Markers

For diagnosis of patients with mild bleeding disorders, analysis of expression of platelet surface glycoproteins is essential. Recent guidelines suggest analysis of GPIIb (CD41, integrin αIIb), GPIIIa (CD61, integrin β_3), GPIbα (CD42b), and GPIX (CD42a) as a first step and GPIa/IIa ($\alpha_2\beta_1$), GPIV, and GPVI as a second step.[56] However, as this review focuses on tests of platelet function, receptor level tests will not be further discussed here. As the surface expression of many of these receptors changes upon platelet activation,[57] these tests should not be performed on activated platelets.

►**Table 1** summarizes different platelet activation events and markers used to detect these. Directly labeled antibodies toward many of these markers are now commercially available, enabling the setup of more advanced

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Table 1 Platelet activation events and markers to detect these

Activation event	Marker(s) with references	Other names, remarks	Possible negative control(s)
Fibrinogen receptor GPIIb/IIIa activation	PAC-1,[29] LIBS/RIBS-specific antibodies,[58–63] fluorescent fibrinogen,[64,65] anti-fibrinogen antibodies[8]	GPIIb/IIIa = αIIbβ$_3$, CD41/61	EDTA-treated platelets, isotype control
Collagen receptor GPIa/IIa activation	Antibody detecting conformational change[66]	GPIa/IIa = α$_2$β$_1$, CD49/CD29	Isotype control
Alpha granule exocytosis	P-selectin,[67–69] CD40L,[70] thrombospondin[71,72]/GMP-33,[73,74] multimerin[75,76]	P-selectin = CD62P, GMP140, PADGEM	Isotype control
		CD40L = CD154	
		GMP-33 = N-terminal fragment of thrombospondin[73]	
Dense granule exocytosis	Mepacrine staining,[77–79] *CD63*,[80] *LAMP-2*[81,82]	CD63 = LIMP, LAMP-3, granulophysin[83]	Nonlabeled platelets (mepacrine), isotype control
		LAMP-2 = CD107b	
Lysosomal exocytosis	LAMP-1,[84,85] *LAMP-2*,[81,82] *CD63*[80]	LAMP-1 = CD107a	Isotype control
PS exposure	Annexin V,[39–41,86] lactadherin[87–89]	Ca^{2+} is required for annexin V binding	Sample without calcium (annexin V)/resting platelets (lactadherin)
Coagulation factor binding	Factor V/Va,[90,91] X/Xa,[92] VIII[93]	Ca^{2+} is required for coagulation factor complex formation[42]	Isotype control
Platelet vesiculation (microparticle formation)	Antibodies toward surface receptors (e.g., GPIIb, GPIIIa, GPIb, GPIX) or annexin V/lactadherin[53] in combination with size characteristics	Better detected with threshold on fluorescence instead of scatter	Resting platelets
Intracellular phosphorylation events	VASP[94]	Designed to measure the function of the platelet ADP receptor P2Y$_{12}$ (more discussed below)	
Changes in intracellular calcium levels	Fluo-3,[95] Fluo-4,[96] Indo-1[97]	Ratiometric measurements are possible by combining dyes with Fura Red[96]	Resting platelets
Changes in mitochondrial membrane potential	A variety of probes are available, based on, for example, carbocyanine (e.g., JC-1), rhodamine (e.g., TMRM) and rosamine (e.g., CMXRos).[98,99]		Resting platelets (for intact membrane), FCCP/CCCP-treated platelets (for fully depolarized membrane)[98,100]

Abbreviations: ADP, adenosine diphosphate; Ca^{2+}, calcium; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CD, cluster of differentiation; CD40L, CD40 ligand; CMXRos, chloromethyl-X-rosamine; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide-4-trifluoromethoxyphenylhydrazone; GMP, granule membrane protein; GP, glycoprotein; JC-1, 5,5",6,6"-tetrachloro-1,1",3,3"-tetraethylbenzimidazolylcarbocyanine iodide; LAMP, lysosomal-associated membrane protein; LIBS/RIBS, ligand/receptor-induced binding site; LIMP, lysosomal integral membrane protein; PADGEM, platelet activation–dependent granule-external membrane protein; PS, phosphatidylserine; TMRM, tetramethylrhodamine methyl ester; VASP, vasodilator-stimulated phosphoprotein.
Note: Markers not specific for one type of granule are shown in italics.

protocols including several markers in the same tube, which is advantageous to reduce the number of tubes if many platelet agonists are to be tested. To minimize problems due to spectral overlap, efforts to choose fluorophores with minimal spectral overlap, careful titration of antibodies, and proper compensation (potentially using capture beads if the antigen expression is low even on strongly activated platelets) are important issues to consider in the protocol setup.[32] Also note that compensation values obtained for platelets do not always match what is commonly reported for larger cells. To determine the boundary between positive and negative platelets, both properly chosen isotype controls and fluorescence-minus-one controls are useful.[31,32] The gate is usually placed to get 1 to 2% positive platelets in the isotype sample.[28] In some cases, such as markers for activated GPIIb/IIIa and annexin V as marker for PS exposure, binding of the marker can be prevented by other means (EDTA treatment[8] and absence of calcium, respectively), making it possible to create a negative control without the use of isotype antibodies.

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Alternative Ways to Test Platelet Function by Flow Cytometry

Other options to study platelet activation using flow cytometry include the agonist-induced formation of platelet microaggregates, detectable by residual platelet counting,[101] the adhesion of platelets to protein-coated polystyrene beads,[102] and the formation of platelet–leukocyte aggregates[103] in circulation or upon platelet activation.[104–106] In studies of platelet–leukocyte aggregate formation, coincidence issues need to be taken into account[107] and distinguished from true aggregates. It also needs to be recognized that platelet–monocyte aggregates form spontaneously after blood collection (see ►**Fig. 1K**), so to be useful in a clinical setting, samples need to be fixed immediately after collection[103] or even in the blood collection tube with fixation added to it.[108]

Measurements of circulating platelet aggregates or P-selectin-positive platelets have been reported in several clinical conditions. However, the rapid shedding of P-selectin on circulating platelets[109] and the potential risk of spontaneous formation after blood collection complicate the interpretation of the results. Protocols including immediate fixation have been described,[110] and might be a possible solution to this problem. However, activated platelets are supposed to be attached to the site of damage and not circulating. Thus, measurement of receptors being cleaved off from activated platelets, such as soluble P-selectin, sGPVI, or sCD40 ligand, might be better alternatives in this context.

Platelet Activation

Platelet activation can be induced by addition of one or more platelet agonists to the diluted blood samples. Agonists can be added in a first separate step or be a part of the antibody mixture in which the blood is diluted. If preincubation with antibodies or inhibitors is needed for optimal performance, agonists can also be added in a subsequent step. Platelet aggregate formation can be avoided by a high enough dilution, gentle mixing, and incubation in static conditions. ►**Table 2** summarizes platelet agonists and receptor activation pathways amenable to testing by flow cytometry. However, it should be kept in mind that release of granule contents is contributing to the activation response by the more potent agonists. ADP receptor inhibition (especially $P2Y_{12}$) and presence of apyrase will therefore decrease platelet responses significantly (see ►**Fig. 1G–I**). The formation of thromboxane A_2 (TXA_2) from arachidonic acid seems to be of less importance, as aspirin treatment is often reported not to affect flow cytometry results.[111–113] The reason for this is somewhat unclear, but the lower cell density and absence of shear might be potential explanations.

The temperature used during activation of platelets will affect the response. The activation step is traditionally performed at room temperature, where higher activation responses are actually seen. It has also been claimed that the PAC-1 antibody is not stable at 37°C,[114] but this is not supported by our data, where preincubation of the antibody at 37°C did not alter its performance if the experiment was performed at room temperature (see ►**Fig. 1J**). Another reason for experiments at room temperature might be that the number of tubes needed for testing of different activation conditions may be cumbersome to handle at 37°C, but it is possible to use a water bath or similar to perform the activation at other temperatures. In the case of annexin V, the binding has been reported to be enhanced at room temperature.[115]

The concentrations of agonists vary between studies, in part due to the choice of anticoagulant, platelet preparation method, etc., but also due to the specific purpose of the study. If the aim is to induce formation of PS-exposing procoagulant platelets, high doses of collagen and thrombin receptor–activating substances in combination are the most effective stimuli,[86] and presence of calcium and autocrine stimulation by ADP[137] are important components.

Studies investigating normal ranges for platelet function in normal donors have shown that the variation is relatively high, especially at low and medium doses of agonists,[26,138,139] and with a considerable overlap in responses between individuals with and without reported bleeding problems. Still, recent large studies of patients with platelet function defects using aggregometry suggest that a streamlined protocol with single doses of several agonists is enough to identify most patients.[140,141] A similar approach might be considered for flow cytometry studies, where a suggestion is to use a dose where the percentage of platelets exposing an activation marker such as P-selectin (for which a full response can be expected) levels out in normal donors.[26] On the other hand, if the aim is to identify individuals with hyperreactive platelets, aggregation studies suggest that submaximal doses are more indicative.[142] Control samples, where platelets are only treated with buffer, should always be included in the protocol to visualize the degree of spontaneous platelet preactivation. This is commonly done by measuring P-selectin exposure, as this is a sensitive marker for preactivated platelets that is not reversible in vitro.[57,143] The level of preactivation should also be reported, and samples showing too high preactivation levels should be interpreted with caution or if possible excluded from evaluation. Information on the appropriate level to use as a threshold for preactivation is scarce. It further depends somewhat on the resolution of the flow cytometer used and of course on the aim of the study. Our experience is that an upper threshold of around 15% P-selectin positive platelets usually identifies samples with an acceptable level of preactivation.

Stopping the Reaction

The activation is ended after an exact time by addition of a larger volume of buffer to stop the reactions. Direct inhibitors to the platelet agonists used, such as hirudin or apyrase, or a fixative may be added to this stopping solution. In clinical settings, a light formaldehyde fixation (0.5–1%) is often used to prolong the sample stability before analysis.[28] However, many fluorophores will show lower intensity, and some antigens may be destroyed by fixation. It is therefore important to test and optimize the protocol before starting a study, and keep conditions constant throughout to allow interpretations and comparisons to be done. As fixation

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Table 2 Summary of platelet agonists and activation pathways possible to test by flow cytometry

Platelet agonist	Receptors	Remarks
Thrombin	PAR1, PAR4, GPIb/V/IX	GPRP peptide needed to prevent fibrin polymerization in PRP/whole blood samples. Do not use with anticoagulants that inhibit thrombin
PAR1-AP	PAR1	The most commonly used and potent peptide has amino acid sequence SFLLRN.[116] Also called "TRAP" or "TRAP-6." SFLLRN also activates PAR2,[117] but this receptor is not present on platelets[118]
PAR4-AP	PAR4	The most used and most active peptide has amino acid sequence AYPGKF, and is based on the mouse PAR4 tethered ligand[119]
ADP	$P2Y_1$, $P2Y_{12}$	Autologous ADP also contributes to the response of other platelet agonists (see ►**Fig. 1G–I**)
CRP-XL	GPVI	Cross-linking is needed for CRP to be active.[120] Fibrillar type I ("Horm") collagen is not an effective agonist in flow cytometry, although methylated acid-soluble collagen type I is more potent[121]
Convulxin	GPVI	C-type lectin isolated from *Crotalus durissus terrificus* snake venom[122]
Epinephrine (adrenaline)	α_2A-adrenergic receptor	Usually only potentiating effects in washed platelet suspensions, but direct effects in PRP and whole blood[123]
U46619[124]	TXA_2 receptor (TP)	PGH_2 analog, potent and stable TP receptor agonist[125]
Rhodocytin,[126] fucoidan[127]	CLEC-2	Podoplanin is the physiological ligand for CLEC-2[128]
Serotonin	5-HT_{2A}	Reported to enhance activation by other agonists in whole blood assays[129]
Arachidonic acid	TP (after conversion to TXA_2 by COX-1 and thromboxane synthetase)	Problems with cell lysis reported, which needs to be checked and proven not to influence the results (Ca^{2+} and plasma may be somewhat protective)[26,130–134]
Ristocetin	Induces conformational changes allowing VWF to bind to GPIb	Has been used to design flow cytometry–based tests for von Willebrand disease.[135] However, problems with cell fragmentation[26] and platelet apoptosis[136] have been reported

Abbreviations: 5-HT_{2A}, 5-hydroxytryptamine receptor 2A; ADP, adenosine diphosphate; AP, activating peptide; Ca^{2+}, calcium; CLEC-2, C-type lectin-like receptor 2; COX-1, cyclooxygenase-1; CRP-XL, cross-linked collagen-related peptide; GP, glycoprotein; GPRP, glycyl-L-prolyl-L-arginyl-L-proline; PAR1/4, protease activated receptor ¼; PGH_2, prostaglandin H_2; PRP, platelet-rich plasma; TP, thromboxane A_2 receptor; TRAP, thrombin receptor activating peptide; TxA_2, thromboxane A_2; VWF, von Willebrand factor.
Note: References are only provided for the more uncommon agonists and for information important to consider when using the different agonists.

affects the platelet membrane properties, it is especially important to verify how fixation affects markers detecting membrane changes, such as PS exposure and formation of microparticles, where fixation may lead to changes wrongly interpreted as being part of the physiological response. In cases where fixation is deemed impossible, flow cytometry analysis needs to be performed as soon as possible, as dilution alone will only keep the samples stable for a shorter period. The maximum time allowed should be tested, applied consistently within the trial participant groups, and reported together with the results.

Analysis

Results from the analysis can be expressed as either the percentage of cells being positive for the marker or the median fluorescence intensity (MFI) of the total platelet population. The percentage of positive platelets has been suggested to be advantageous in situations where all cells do not become positive,[28,144] and also due to its assumed transferability between instruments, but only if proper controls are applied to set the boundary between negative and positive platelets, as discussed above. The other alternative is the MFI, which reports the median signal from bound antibody for the total platelet population. This approach will be advantageous in situations where most platelets express the antigen, but where the level of expression could vary between individuals[21] or treatments. The geometric mean is an alternative to the median. The mean should not be used as a few highly positive events are often detected, which tend to make the mean unreliable. However, the drawback with MFI in a large clinical study with an extended patient recruitment or follow-up period is that the flow cytometer performance may shift with time. Thus, the instrument stability over time needs to be verified for the results to be comparable. Control beads of different kinds are provided by the manufacturers to verify linearity and detector/laser performance over time,

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and should always be used to monitor instrument performance. As the MFI scaling is arbitrary, it is necessary to run fluorescence calibration beads allowing responses to be reported as Molecules of Equivalent Soluble Fluorochrome (MESF),[145] if results are to be compared between instruments and over time in, for example, multicenter trials.

For clinical studies aiming to identify and/or diagnose patients with platelet dysfunction, it is essential to establish appropriate reference intervals. The guidelines from the British Society of Haematology for investigation of heritable platelet function disorders recommend that local, normal cutoff values are established using nonparametric statistics, but recognizes that this is not commonly done.[22] Indeed, the intra- and interindividual variation and the presence of high and low responders among normal donors make this a challenging task. Also, samples from 120 individuals are recommended to create a reference interval using nonparametric methods.[146] However, other approaches such as bootstrap-based procedures[147] or the robust method described by Horn and Pesce[148] may enable calculation of reference intervals with a lower number of reference samples.[146,149]

Current Use of Flow Cytometry for Platelet Function Testing In Clinical Settings

Diagnosis of Mild Bleeding Disorders

A survey regarding the diagnosis of patients with mild bleeding disorders including 202 laboratories in 37 countries was recently reported by the International Society of Thrombosis and Haemostasis.[150] A flow cytometer was available in 57% of the laboratories. It was used as a first step test in 23% and as a second step test in approximately two-thirds of the laboratories. The main use was to study expression levels of receptors known to be absent in rare inherited platelet disorders such as Glanzmann thrombasthenia and Bernard–Soulier syndrome. Seventy-eight of the laboratories used flow cytometry to study α granule release, measuring P-selectin exposure upon activation. In addition to that, activation of fibrinogen receptor GPIIb/IIIa was relatively common, but only using a few agonists, often ADP and PAR1-AP (TRAP-6), while other tests, such as dense granule release, procoagulant activity, or lysosomal release, were assessed by less than 30 of the laboratories. Light transmission aggregometry was available in 73% of the laboratories and used for first-line testing in 59%. Notably, out of an estimated number of 14,000 patients investigated each year, no abnormality was found in 60% of the cases, and only 9% of the remaining received a definite molecular diagnosis. This indicates that the current battery of tests may be insufficient to indicate where a potential defect is located.

Transfusion Medicine

Flow cytometry has been commonly used in transfusion medicine to investigate the in vitro quality of platelets in platelet concentrates during storage and to assess how different preparation and storage conditions affect platelet properties.[151–154] However, most studies have not included any addition of platelet agonists, but studied changes in spontaneous expression of receptors such as GPIb[152,155] and GPIIb/IIIa,[152,156] P-selectin[154,155] or CD63[152,155] as activation markers and annexin V as apoptosis marker.[152] Spontaneous increases in P-selectin exposure, annexin V binding, and CD63 are often reported, while the receptor expression studies have been showing variable results. Even less is known about the agonist-induced expression and how it is affected by storage. The upregulation of GPIb (CD42b) and P-selectin after agonist stimulation has been found to decrease during storage,[157–160] as well as the PAC-1 expression.[161] Agonist-induced formation of microparticles and exposure of LAMP-1 was recently suggested as potential new markers to detect deteriorating platelet function during storage.[54] How these findings correlate to in vivo performance of transfused platelets is, however, still unknown. Only a few studies have investigated the correlation between expression of activation markers and in vivo viability and the correlations reported so far have been low and varying between studies.[162–164] Thus, investigation of the platelet activation potential using flow cytometry could potentially contribute to better results in this regard.

Anti-Platelet Drug Testing

One commercial flow cytometry–based test has been developed to measure the function of the platelet $P2Y_{12}$ receptor, with the aim to study the effect of anti-platelet drugs targeting this receptor (e.g., clopidogrel, prasugrel, ticagrelor). In the vasodilator-stimulated phosphoprotein (VASP) assay, platelets are incubated with either prostaglandin E_1 (PGE_1) or ADP + PGE_1, and the phosphorylation state of VASP is measured to determine the degree of active $P2Y_{12}$ receptor. The test has been compared with other approaches to measure $P2Y_{12}$ inhibition in clinical settings, but the frequency of "nonresponders" identified has been shown to vary considerably depending on the assay performed.[165] In addition, the correlations between the assays have been low in several studies.[166] As an alternative to the flow cytometry protocol, an enzyme-linked immunosorbent assay method for VASP phosphorylation has been developed. This enables testing of plasma samples, thereby making remote testing easier to perform.[167] Direct or indirect methods to detect platelet GPIIb/IIIa antagonists, as well as methods based on arachidonic acid to detect aspirin use, have been described, but none has gained widespread attention or commercial use. A recent study comparing flow cytometry to other methods to determine platelet function in patients on dual antiplatelet therapy reports low correlations between the different arachidonic acid–based methods.[168] The above-mentioned potential issues with cell lysis by arachidonic acid could be one explanation, but that needs to be further investigated.

Thrombosis and Disease Risk Assessment

The potential to form procoagulant platelets upon strong activation with thrombin in combination with convulxin or collagen has been measured in several disease states. Increased formation of procoagulant platelets (measured as coated platelets) has been reported in thrombotic conditions such as ischemic stroke and transient ischemic attack[169–172] and lower formation in hemorrhagic conditions such as

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hemorrhagic stroke.[173–175] However, as these reports originate from a single center, additional studies from independent groups are needed to strengthen these findings. Platelet function tested by flow cytometry was also recently reported to be associated with bleeding severity in children with immune thrombocytopenia independent of the platelet count.[176]

Although not platelet function tests, and with the limitations discussed previously, circulating P-selectin-positive platelets and platelet–leukocyte aggregates have been described in several conditions as reviewed by Linden et al,[177] with platelet–leukocyte aggregates being potentially more informative, as P-selectin is rapidly removed from the surface of activated platelets in vivo.[109]

Flow Cytometry for Platelet Function Testing in Clinical Settings—New Perspectives

►**Table 3** summarizes advantages and challenges for platelet function testing by flow cytometry in a clinical setting. The major advantage is the possibility to test several different aspects of platelet function in a small volume of whole blood and also in thrombocytopenic patients. This together with the increasing availability of flow cytometers and improvements in sample preparation, including new fixation possibilities and premade tubes which allows remote site testing,[108,110,178,179] opens up interesting future avenues. Especially in the field of mild bleeding disorders, it will be interesting to see if light transmission aggregometry in the future is challenged by flow cytometry for the diagnosis of these patients. Bleeding risk assessment in patients with thrombocytopenia is another interesting future possibility.

For assessment of thrombotic events, the situation is more complicated, as the mechanisms underlying these events are more complex. In case of persistent hypercoagulability or vessel wall problems, it is also important to keep in mind that all ex vivo platelet function studies will only target platelets that are still circulating. This means that a lower platelet activation response could be expected. Indeed, this was the case in a recent study by us, where lower platelet activation was found in circulating platelets in patients infected with hantavirus with ongoing platelet destruction.[180] For antiplatelet drug testing or in acute settings, flow cytometry may be less useful, as the turnaround time might still be considered too long, despite the improvements regarding sample preparation mentioned above. Furthermore, the potential to use multicolor protocols also means that the data analysis and its interpretation will become increasingly complex. A future challenge may then be to develop and evaluate multiparameter analysis algorithms with the potential to capture and identify defects and features of platelet functionality that may have diagnostic and/or prognostic potential.

Concluding Remarks

Flow cytometry allows a versatile and sensitive simultaneous testing of a multitude of platelet properties and functions. The high sensitivity requires knowledge of the level of platelet preactivation to be able to interpret the results. But on the other hand, flow cytometry is one of few platelet function tests where this is easily achieved by inclusion of a buffer-treated sample. The method works in thrombocytopenic patients and only requires low volumes of blood. And even though traditional platelet function testing by flow cytometry requires skilled staff and therefore is impractical in many clinical settings, newly introduced and future improvements may well be paving the way for more widespread clinical use. The increasingly challenging analysis needs to be dealt with before this can become reality. In addition, there is a need for studies coupling flow cytometry test results to clinical outcomes, and also comparative studies with other tests.

Table 3 Summary of advantages and limitations/challenges in the development of flow cytometry platelet function testing for clinical use

Advantages
• Tests can be designed to include many different aspects of platelet function and surface expression in a single protocol • Low volumes of blood are needed, which is advantageous in, for example, pediatric patients • Whole blood can be used, thereby avoiding the risk of losing denser platelet populations or causing preactivation by centrifugation steps • Platelet function can be evaluated in thrombocytopenic patients • Platelet preanalytical activation can be easily tested and accounted for • The field is still in its early days. Thus, it seems possible that many researchers and diagnostic laboratories could adopt a well-designed and standardized protocol
Limitations/challenges
• Only cells in diluted suspensions can be used, so it is not possible to study aspects/signaling induced by platelet–platelet contacts • An increasing number of flow cytometers on the market increase the need for standardization efforts • The preparation of samples is labor-intensive (but with potential improvements) and a skilled operator is needed to analyze the obtained results • Many proposed protocols are not yet validated by studies of patients with well-defined defects • End-point measurements are usually the only option • Does not include any shear stress component

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